J Mol Evol (1992) 35:486–491

Journal of
Molecular Evolution

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Nucleotide Variation and Molecular Structure of the Heterochromatic Repetitive AluI DNA in the Brine Shrimp *Artemia franciscana*

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Summary. It has been suggested that DNA bending could play a role in the regulation of gene expression, chromosome segregation, specific recombination and/or DNA packaging. We have previously demonstrated that an AluI DNA family of repeats is the major component of constitutive heterochromatin in the brine shrimp *A. franciscana*. By the analysis of cloned oligomeric (monomer to hexamer) heterochromatic fragments we verified that the repetitive AluI DNA shows a stable curvature that determines a solenoidal geometry to the double helix. This particular structure could be of relevant importance in conferring the characteristic heterochromatic condensation. In this paper we evaluate how the point mutations that occurred during the evolution of the AluI sequence of *A. franciscana* could influence the sequence-dependent tridimensional conformation. The obtained data underline that, in spite of the high sequence mutation frequency (10%) of the repetitive DNA, the general structure of the heterochromatic DNA is not greatly influenced, but rather there is a substantial variation of the copy number of the repetitive AluI fragment. This variation could be responsible for the hypothetical function of the constitutive heterochromatin.

Key words: Brine shrimp — AluI DNA — Nucleotide variation

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Introduction

Highly repetitive DNAs (satellite DNA) (Brutlag 1980; Singer 1982) often comprise a substantial portion of the eukaryotic genome and are mainly localized in the constitutive heterochromatin in an apparently genetically inert and compact part of the chromosomes (Bernard 1988). A characteristic of repetitive DNAs is their variability in type and abundance for closely related species (Hennig 1972; Mazrimas and Hatch 1972); nevertheless, identical satellite DNA sequences were observed in a great number of species characterized by a large evolutive divergence (Salser et al. 1976; Fry and Salser 1977; Peacock et al. 1977). The species specificity of satellite DNA was tentatively explained by Salser et al. (1976), who proposed the existence of a sequence library common also to distantly related species. Amplification of different members of the library would yield a species-specific satellite DNA complement, resulting in rapid evolutionary fluctuations in satellite profiles, yet allowing conservation of the satellite sequences themselves. This conservation would be considered evidence for physiological functions (Avila et al. 1983; Beridze 1986; Gatti et al. 1983; Pardue and Hennig 1990; Karpen and Spradling 1990; Hayashi et al. 1990; Wilson et al. 1990). Utilizing the brine shrimp *Artemia* as a biological system we are attempting to contribute to the understanding of the physiological role of constitutive heterochromatin, supposing that the particular structure caused by a nucleotide sequence to satellite DNAs (Benfante et al. 1989; Martines-Balbas et al. 1990) can be the key for understanding

its function(s). The genus *Artemia* includes a set of sibling species defined by reproductive isolation. Endemic in the Old World are the bisexual *A. salina* (in the Mediterranean region), *Artemia* species from China and also the parthenogenetic populations distributed in Europe, Africa, and Asia. Endemic in the New World are *A. persimilis* limited to Argentina and *A. franciscana* represented by populations which are geographically isolated, but still interfertile. The finding of a clustered repetitive AluI DNA family of 113 bp localized in the heterochromatin close to telomeres of *A. franciscana* has been described (Barigozzi et al. 1984; Cruces et al. 1986), and its distribution has been studied in 13 strains, including bisexual species and parthenogenetic populations. These analyses revealed that the copy number of the AluI sequence is higher in the two bisexual species *A. persimilis* and *A. franciscana* compared with the remaining *Artemia* species and parthenogenetic populations which show a very low amount or a virtual absence of the AluI repeats (Badaracco et al. 1987, 1991). We have recently shown that the AluI DNA fragment is characterized by an intrinsic curvature determined by the nucleotide sequence and that monomeric units belonging to the same cluster have a direct tandem organization. This suggests that the curvature of each monomer could be additive, conferring a specific solenoidal structure to the repetitive DNA responsible for the heterochromatic condensation and probably for its role in the cell (Benfante et al. 1989). In this paper we have studied the evolution, at qualitative and quantitative levels, of the heterochromatin present in *A. franciscana*. The results show that while the structure is not substantially modified, great variation occurs in the copy number of the AluI monomer present in the cells of different populations, suggesting that the hypothetical function(s) of the constitutive heterochromatin could be modulated by its quantitative variations.

Materials and Methods

Artemia Strains. The biological material analyzed in this investigation was mainly provided as dry cysts by the Laboratory of Mariculture (Artemia Reference Center) of the University of Ghent, Belgium. The strains of *A. franciscana* studied are from: Chaplin Lake (Canada); Great Salt Lake (Utah, USA); San Francisco Bay (California, USA); Yucatan (Mexico); Porto Virrile (Peru); Araya (Venezuela); and Cabo Frio (Brazil).

DNA Cloning. Genomic DNA of the indicated *A. franciscana* populations was extracted from living nauplii grown as previously described (Badaracco et al. 1987). The monomeric and multimeric fragments of heterochromatic DNA were obtained by a partial AluI digestion of the purified genomic DNAs (Benfante et al. 1989). Fragments were separated on 1% agarose gel, eluted, and then cloned according to Maniatis et al. (1982) into the SmaI site of pUC18, where the HindIII site of the polylinker was replaced by an EcoRI site (pUC18m; Venditti et al. 1988). Positive recombinants were identified by colony hybridization using the cloned AluI monomer as probe (Barigozzi et al. 1984). The inserts were sequenced by the dideoxy-nucleotide chain-termination method (Sanger et al. 1977).

Gel Electrophoresis. The mobility shift electrophoresis of the multimeric fragments was performed on 10% polyacrylamide gel in 40 mM Tris · HCl (pH 7.8), 20 mM sodium acetate, 2 mM EDTA for 18–20 h at 2.5 V/cm at 4°C with recirculation of the buffer. Gel was analyzed by ethidium bromide staining.

DNA-Blot Procedure and Hybridization. DNA was dotted onto nitrocellulose filter. The filter was prehybridized and hybridized as previously described (Badaracco et al. 1987).

Results

Sequence Variation of the AluI repetitive DNA in the Artemia Population From San Francisco Bay

In order to evaluate the intrapopulation variability of the heterochromatic AluI DNA sequence and therefore its structural modifications, we sequenced several recombinant plasmids containing cloned monomeric and oligomeric heterochromatic fragments of the artemia population from San Francisco Bay. The results show that, on 17 monomers investigated, the sequence variation is quite high (between 0 and 10%) not only for monomers possibly belonging to different chromocenters, but also for repetitive units of the same cluster. This was in fact verified by sequencing two 2-mers, four repeating units of a 5-mer, and four repeating units of a 6-mer (Fig. 1). As is easily observed, mutations, which are exclusively base substitutions, are not randomly distributed along the AluI monomeric sequence; rather they map mainly between nucleotides 5 and 13 and nucleotides 40 and 60. In all cases the substitutions occur outside of the eight blocks of A or T that characterize the heterochromatic units determining the solenoidal structure (Benfante et al. 1989), the only exception being the block at nucleotides 54–56, which can be interrupted by a G.

Structural Analysis of the Heterochromatic DNA Fragments From Artemia Population Living in San Francisco Bay

The structural variations of the heterochromatic fragments, dependent on the nucleotide sequence, were verified by evaluating their K-factor (Benfante et al. 1989).

Figure 2 shows the pattern, on a 10% polyacrylamide gel, of a partial AluI digestion (lane 2) of the EcoRI fragment (lane 3) obtained by a plasmid car-

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INTRAPOPULATION VARIABILITY
         10        20        30        40        50        60        70        80        90       100       110
         I         I         I         I         I         I         I         I         I         I         I
GATGCATACAACCTTTTTACTTCTATAATCTGATAAAATACTAACGTGTACCATTTCTTACACTAATTTCCGAGAAAAGGATACAGTTTTCAAAATCAAAAGCTCCCATTATC   1
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INTERPOPULATION VARIABILITY

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Fig. 1. Sequence analysis of AluI repetitive DNA from several populations of *A. franciscana*. The figure shows the alignment of the following sequences obtained from AluI repetitive DNA of artemia population from San Francisco Bay. The first five sequences are monomers of independent clones, while monomers 6, 7; 8, 9; 10–13; and 14–17 belong respectively to two 2-mers, a 5-mer, and a 6-mer. Sequences 18–20; 21–23; 24, 25; 26–28; and 29, 30 are monomeric units cloned from genomic DNA of *Artemia* populations respectively from Chaplin Lake (Canada); Great Salt Lake (Utah, USA); Yucatan (Mexico); Porto Virrile (Peru); and Cabo Frio (Argentina). ○ and ø symbols represent respectively nucleotide residues belonging or not to the blocks of three or more adenines or thymines; the letters indicate the substitutions observed with respect to a monomeric unit arbitrarily assumed to be standard sequence (1).

rying a 5-mer heterochromatic insert. The scheme represents the pattern of the restricted fragments, including the polylinker sequences joined to the border of the heterochromatic fragments identified by Southern blot hybridization (data not shown). The result clearly demonstrates a heterogeneity in the migration, not only for the fragments containing the polylinker sequences, but also among the AluI fragments, and therefore suggests a heterogeneity of their molecular geometry. The K-factors of the 5-mer shown in Fig. 2 and also that of a 4-mer are listed in Table 1 and show a variability ranging between 1.13 and 1.43 both for monomers as well as for dimers. Heterochromatic fragments terminating with polylinker sequences usually present a higher K-factor. This could be due to an increase in steric hindrance occurring when a straight molecule is joined to bent structure or be due simply to the longer sequence of the fragments.

The experiment demonstrates that the variability of sequences flanking the blocks of A or T influences the bending of the monomeric unit even if the general geometry of the cluster is substantially maintained.

Sequence and Structural Analysis of the AluI Repetitive DNA Units From Different Populations of A. franciscana

Having determined the intrapopulation variability of the AluI heterochromatic fragments, we analyzed 13 AluI monomers cloned from five other different populations of *A. franciscana*, distributed on a large part of the American continent.

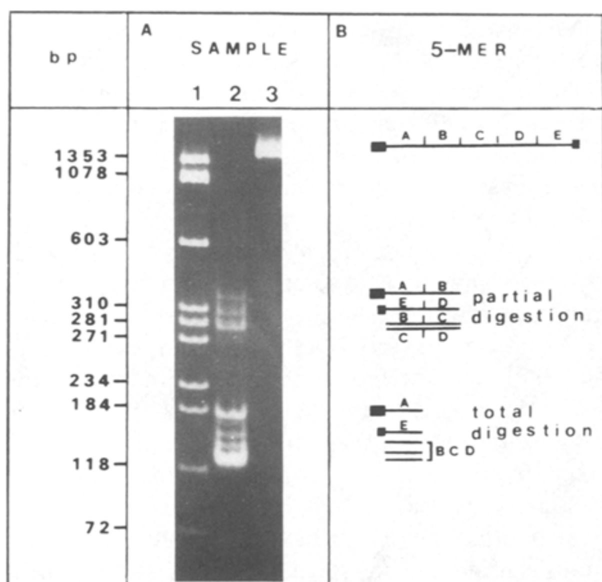

Fig. 2. Electrophoretic mobility of cloned monomeric and multimeric AluI DNA fragments. Panel **A** shows the migration of fragments obtained by partial and total digestion with EcoRI and AluI restriction enzymes of plasmids containing a pentamer insert (lane 2). Lane 3 shows the migration of the undigested 5-mer. Lane 1 represents the migration of 1 μg of φX174 DNA digested with HaeIII as a control of molecular length. The products of the digestion of the 5-mer are represented respectively in panel **B.** Heterochromatic units are schematically indicated by a thin line, while thick lines indicate the polylinker sequences left by the EcoRI digestion.

The sequences (reported in Fig. 1, lanes 18–30) do not differ greatly, in terms of number and position of nucleotide substitutions, from those examined in the San Francisco Bay population.

The same result (Fig. 3) is given by the acrylamide gel electrophoretic analysis of the monomers obtained by the EcoRI digestion of the different recombinant plasmids. The calculation of the relative K-factors (Table 2) confirms the assumption that the heterochromatic DNA distortion is maintained. In fact, the K-values range between 1.29 and 1.54, and this difference is in accordance with that obtained in the artemia population from San Francisco Bay (Table 1).

Quantification of the AluI Family of Repeats in Different Populations of A. franciscana

The cloned AluI DNA fragment of *Artemia* from San Francisco Bay was used as probe in a dot-blot hybridization experiment to quantify the AluI repetitive sequence present in different populations of *A. franciscana* distributed on the American continent. As shown in Fig. 4, the AluI repetitive DNA content varies greatly in the populations tested with an evident increase from south to north.

Table 1. Analysis of the distortion of heterochromatic cloned DNA of *A. franciscana* from San Francisco Bay.

Artemia population (clone)	bp	Apparent length	K-factor[a]
San Francisco Bay (5-mer)			
partial digestion	616	1400	2.30
dimer A-B	261	375	1.43
dimer E-D	240	300	1.25
dimer B-C, dimer C-D	226	270	1.20
total digestion			
monomer A	148	185	1.25
monomer E	127	160	1.25
monomer B, monomer C, monomer D	113	128–140	1.13–1.23
San Francisco Bay (4-mer)			
total digestion	503	1010	2.00
monomer F	148	185	1.25
monomer K	127	175	1.37
monomer G, monomer H	113	155, 128	1.37, 1.13

[a] K-factor was calculated as the ratio of the apparent length of the fragment, evaluated on polyacrylamide gel electrophoresis, to the length found with sequence analysis

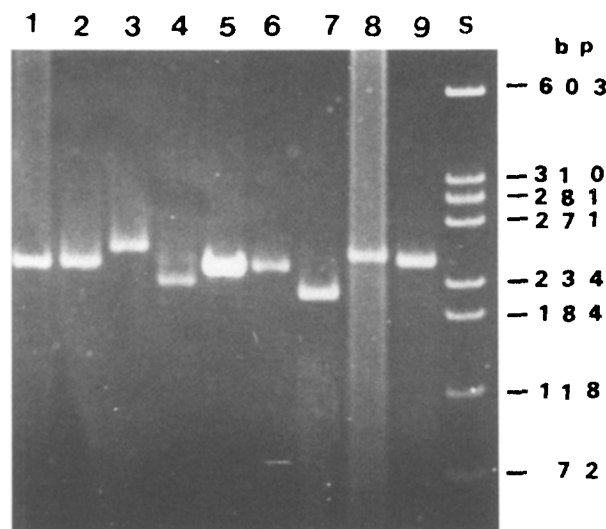

Fig. 3. Electrophoretic mobility of cloned monomeric fragments from several populations of *A. franciscana*. The figure shows the migration of EcoRI fragments of plasmids containing the monomeric insert of heterochromatic DNA of populations from Chaplin Lake (lanes 1, 2); Great Salt Lake (lanes 3, 4); San Francisco Bay (lane 5); Yucatan (lane 6); Porto Virrile (lanes 7, 8); and Araya (lane 9). Lane S represents the migration of φX174 DNA-HaeIII fragments utilized as markers of molecular length.

Discussion

Previous studies (Badaracco et al. 1987, 1991) demonstrated that an AluI DNA family of repeated sequence of 113 bp in length is the major component of the heterochromatin in *A. franciscana*.

Table 2. Analysis of the K-factor of monomeric heterochromatic fragment cloned from different populations of *A. franciscana*

Artemia population (clone)	bp	Apparent length	K-factor
Chaplin Lake (D2)[a]	162	245	1.51
Chaplin Lake (D3)	162	248	1.53
Great Salt Lake (A17)	162	210	1.29
Great Salt Lake (A30)	162	240	1.48
San Francisco Bay (N1)	162	235	1.45
Yucatan (C1)	162	230	1.42
Porto Virrile (F1)	162	250	1.54
Porto Virrile (E8)	162	242	1.49
Araya (G3)	162	242	1.49

[a] In parentheses are indicated the used clones.

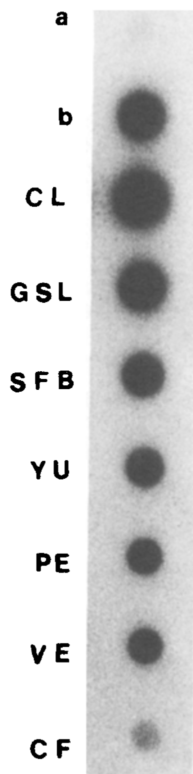

Fig. 4. Evaluation of the amount of AluI repetitive DNA in different populations of *A. franciscana*. Genomic DNAs were prepared as described in the legend of Fig. 1 and dot-blotted (1 μg each) on nitrocellulose filter. Hybridization was carried out as described by Badaracco et al. (1987). The populations of *Artemia* analyzed are from Cabo Frio (CF), Araya (VE), Peru (PE), Yucatan (YU), San Francisco Bay (SFB), Great Salt Lake (GSL), and Chaplin Lake (CL). Small *a* represents 1 μg of calf tymus DNA and small *b* 100 ng of pUC18m plasmid containing the cloned AluI repetitive unit.

On the basis of the analysis of cloned oligomeric (monomer to hexamer) AluI fragments we revealed (Benfante et al. 1989) that the sequence produces a stable curvature of the axis of the heterochromatic DNA. Moreover, we demonstrated that the unusual conformation of the heterochromatic fragments can be modulated in vitro when the DNA samples are treated with distamycin and that the drug can also produce a visible decondensation of the heterochromatic masses of the interphase nuclei in vivo (Benfante et al. 1989).

The observation that heterochromatic DNA could be made up as a solenoidal structure and that condensation becomes incomplete in the presence of distamycin in vivo (Radic et al. 1987; Benfante et al. 1989) seems to indicate an important role of the DNA curvature in the heterochromatin structure organization and perhaps in its function.

On the basis of these considerations we have investigated, in this paper, whether the structure of heterochromatic DNA has undergone consistent modifications during speciation. The results show that the nucleotide mutation rate of the AluI repetitive sequence in different populations of *Artemia* is quite high (reaching 10%) even if this variation does not differ greatly from the intrapopulation variability verified also inside the same cluster.

Our experiments demonstrate that the nucleotide substitutions almost never occur at blocks of A or T, but slightly influence the bending of the monomeric units. The solenoidal geometry of the cluster, however, is substantially maintained. In a previous report (Badaracco et al. 1987) we described a quantitative variation of AluI DNA in four populations of *A. franciscana*. The analysis on seven populations of *A. franciscana* (Fig. 3) confirms that observation and points out the existence of a gradient of repetitive DNA concentration, increasing from the south to the north of the American continent, with a variability higher than 100 times.

In conclusion, the described observations seem to indicate that during evolution the general plain of the intrinsic structure of the heterochromatic DNA has not been greatly influenced by nucleotide substitutions occurring in the AluI repetitive sequence, and that its hypothetical function(s) could be determined and/or modulated through quantitative variations of the repetitive AluI units' content.

Acknowledgments. This research was supported by the National Research Council of Italy, Special Project RAISA subproject 3, paper 580, and Piano Finalizzato Ingegneria Genetica (grant N. CT 89.03380.14.115.02867).

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Received March 6, 1992/Revised May 4, 1992